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Eutrophication may compromise the resilience of the Red Sea coral *Stylophora pistillata* to global change

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ABSTRACT

Environmental stressors are adversely affecting coral reef ecosystems. There is ample evidence that scleractinian coral growth and physiology may be compromised by reduced pH, and elevated temperature, and that this is exacerbated by local environmental stressors. The Gulf of Aqaba is considered a coral reef refuge from acidification and warming but coastal development and nutrient effluent may pose a local threat. This study examined the effects of select forecasted environmental changes (acidification, warming, and increased nutrients) individually and in combination on the coral holobiont *Stylophora pistillata* from the Gulf of Aqaba to understand how corals in a potential global climate change refugia may fare in the face of local eutrophication. The results indicate interactions between all stressors, with elevated nutrient concentrations having the broadest individual and additive impacts upon the performance of *S. pistillata*. These findings highlight the importance of maintaining oligotrophic conditions to secure these reefs as potential refugia.

1. Introduction

Coral reefs world-wide are challenged by global environmental change, primarily global warming and ocean acidification (Hoegh-Guldberg et al., 2017). Many reefs of the Pacific and Atlantic are already in a bad state due to the combined effect of global (warming and acidification) and local stressors (Hoegh-Guldberg et al., 2007; Hughes et al., 2007; Knowlton and Jackson, 2008) making the future of previously diverse and functional reefs, uncertain (Pendelton et al., 2016). Predictions about the impact of global environmental change on coral reefs is often based on experiments using one or two factors on a single species (but see Dove et al., 2013), rarely taking a multi-factor approach (Pendelton et al., 2016).

Alongside global deterioration in oceanic environments from warming and ocean acidification, local disturbances such as overfishing, mining, nutrient runoff, and pollution are likely to lower the resilience of corals to environmental change (Burke et al., 2011; Kennedy et al., 2013). Eutrophication occurs with increased inputs of nutrients from the production and use of chemical fertilizers, discharges of human and animal wastes, atmospheric NO_x inputs from fossil fuel burning, and upwelling events (Galloway et al., 2008). Nutrients are critical in supporting reef systems; however, nutrients in excess can lead to the destruction of reefs (D'Angelo and Widenmann, 2014). Nutrient enrichment (primarily nitrogen and phosphorus) can cause shifts in trophic dynamics of coral reef ecosystems (Szmant, 2002), loss of coral cover and diversity (Fabricius, 2005), and increased susceptibility to bleaching (Wiedenmann et al., 2012). Increased nutrients can also lead to increased benthic macroalgae cover, which can out-compete corals for available substrate, act as a sediment trap, smother corals, and reduce the depth of light penetration (McCook et al., 2001; Belliveau and Paul, 2002; Beman et al., 2005). However, effects of elevated nutrients are often coral species specific and depend on the type and ratio of nutrients exposed to the corals (Bucher and Harrison, 2002; Wiedenmann et al., 2012).

Predicted declines in reef ecosystem health from increased nutrients could be exacerbated by increasing atmospheric CO_2 levels and resulting warming and ocean acidification (Feely et al., 2009; Kleypas and Yates, 2009; Chauvin et al., 2011; DeCarlo et al., 2015). The combined effect of elevated temperature, reduced pH, and elevated nutrients may further detrimentally affect coral reef trophic interactions, yet these

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potential interactive effects are not currently well-understood. Indeed, the present data on the combined effects are often contradictory. For example, Renegar and Riegl (2005) showed that effects of increased pCO_2 on growth rates of *Acropora cervicornis* were greater than nutrient enrichment alone, however high concentrations of nitrate or phosphate resulted in significant decreases in growth rate in both the presence and absence of increased pCO_2. Yet Langdon and Atkinson (2005) and Holcomb et al. (2010) demonstrated that elevated levels of nitrate, ammonia, and phosphate rendered some species of coral less sensitive to the effects of elevated CO_2. In addition, Marubini and Atktinson (1999) demonstrated that calcification rates of corals (*Porites compressa*) decreased under reduced pH but was not affected with the addition of elevated nitrate. There are also suggestions that corals may benefit from the addition of nutrients (phosphorus) that may protect corals during a bleaching event (Ezzat et al., 2016; Rosset et al., 2017). Alternatively, bioerosion of some corals is increased in elevated CO_2 environments and is ten times greater under high nutrient conditions (DeCarlo et al., 2015). Hence, elevated nutrients can act as a confounding factor that can influence coral response to an altered state of acidification and lead to ambiguous interpretations.

Corals are a complex holobiont consisting of the animal and its associated suite of internal and external organisms (Rosenberg et al., 2007). Environmental stressors such as increasing frequency and intensity of thermal anomalies, ocean acidification, and elevated nutrients, may cause disturbances or shifts in the composition of the holobiont (Hoegh-Guldberg et al., 2007; Bourne et al., 2009; Mouchka et al., 2010). These environmentally driven shifts in the *Symbiodinium* and microbial communities can detrimentally affect coral health (Sweet et al., 2010). Therefore, it is important to understand the effects of these stressors on major members of the holobiont.

The Gulf of Aqaba is a semi-enclosed water body located at the northern end of the Red Sea. Situated in an arid area, and given that the shallow Bab el Mandeb straits (137 m) allow only nutrient deplete surface waters to enter the Red Sea, corals in the Gulf of Aqaba are exposed to exceptionally low nutrient concentrations (Klinker et al., 1978; Badran et al., 2005; Manasrah et al., 2006). Even so, increasing temperatures, ocean acidification, and nutrient inputs through upwelling (Genin et al., 1995; Labiosa et al., 2003), fish farming (Bongiorni et al., 2003; Kramarsky-Winter et al., 2009; Laiolo et al., 2014), and poorly managed coastal wastewater facilities (Naumann et al., 2014) are noted to have deleterious effects on local coral reefs. For example the nutrient enrichment caused by an atypically deep upwelling that occurred due to the anomalously cold winter following the eruption of Mount Pinatubo in 1991, caused an extensive phytoplankton and benthic algal bloom (Genin et al., 1995). The subsequent smothering of corals by an *Enteromorpha* sp. algal mat resulted in 20.5% mortality of branching coral species (Genin et al., 1995). Similarly transplantation of brooding coral colonies to a nutrient enriched fish farm site significantly reduced the number of planulae produced compared with control colonies (Loya, 2004). Despite the significant impact which nutrient enrichment appears to have, no controlled experiments tested the combined effects of local and global scale stressors in the Gulf of Aqaba. We therefore performed a multifactorial experiment on the scleractinian coral *Stylophora pistillata* which is commonly found in the Gulf of Aqaba. Specifically, we sought to determine the effects of each environmental variable (warming, ocean acidification, and eutrophication) in isolation and in combination on the coral holobiont performance to better inform predictions of coral's response to future environmental changes.

2. Methods

2.1. Experimental design

In October 2013, four colonies of *Stylophora pistillata* were collected from a coral nursery at 8 m depth (29° 30′ N, 34° 56′ E) opposite the Interuniversity Institute (IUI) for Marine Sciences in Eilat, Israel (Gulf of Aqaba). The colonies were immediately sectioned into fragments, labeled by genotype, and acclimated in running seawater for 30 days. The full multifactorial design consisted of 8 different combinations of pH, temperature, and nutrients (CCC – control, CCH – high nutrients, CLC – low pH, HCC – high temperature, CLH – low pH + high nutrients, HCH – high temperature + high nutrients, HLC – high temperature + low pH, HLH – high temperature + low pH + high nutrients). Each treatment was replicated 4 times. One fragment from each genotype was placed in every flow-through experimental aquarium using the Red Sea Simulator at the IUI (Krueger et al., 2017; Bellworthy and Fine, 2017). This state of the art flow-through system was specifically designed to hold organisms for extended periods of time under climate change and ocean acidification conditions and is equipped with a high precision nutrient dosing apparatus. For the purposes of this study, seawater was pumped in from the Gulf of Aqaba, filtered, and pH was adjusted in 2000 L mixing tanks. Temperature was controlled by heat exchange systems in each individual aquarium.

CO_2 gas was bubbled into two mixing tanks to decrease seawater pH. Well-mixed filtered (500 μm) seawater continuously flowed from the mixing tanks into the corresponding test aquarium tanks. A pH probe (SeaFet, Satlantic, Halifax, Canada) reported pH of reef water to the system (ca. 8.1) and the set value of the pH in the aquariums was a delta from that pH value (−0.3 pH units, ca. 7.8). Daily pH variability was in the range of ± 0.02 pH units. Temperature was set as a delta from reef water temperature (ambient ca. 24 °C and high temperature + 5 °C, ca. 29 °C) and controlled by a complex set of heat exchangers capable of keeping water temperature within a range of 18–34 °C regardless of the ambient seawater temperature or weather conditions. A two-armed robot, equipped with pH, temperature and oxygen probes on each arm, monitored each aquarium sequentially ca. once an hour. Using only two sets of probes on all treatment aquariums decreased the probability of discrepancy among probes and errors arising from probe calibration. Prior to the experiment, the pH electrodes were calibrated against National Bureau of Standards (NBS) scale buffers of 4.01, 7.00, and 10.00 at 25 °C. All holding tanks and the robot were controlled with software (Crystal Vision, Samar, Israel) to monitor and control pH and temperature. An elevated (relative to Gulf of Aqaba concentrations) nutrient mixture at Redfield Ratio (nitrate + nitrite (1 μM) + phosphate (0.0625 μM)) was continuously dosed into the high nutrient treated aquarium tanks at a rate of 0.5 L/min using GHL dosing systems with integrated computers. These concentrations were chosen based on the highest nutrient concentrations seen in the Gulf of Aqaba (Klinker et al., 1978; Korpal, 1991; Al-Qutob et al., 2002; Manasrah et al., 2006). Nutrients (NO_3 and PO_4) were monitored at initial dosing, halfway, and at the termination of the experiment. Nutrient concentrations were determined using colorimetric techniques with a QuikChem 8000 flow injection autoanalyzer (Lachat Instruments, Milwaukee, USA).

To avoid direct sunlight on the corals, a wave generator pump agitated the water surface and incident light and was monitored above and underwater using a Kipp and Zonen CM11B light meter (average daily light maximum was 1375.7 ± 7.1 S.E.). The coral fragments were maintained in pH and temperature treatments for 30 days and nutrient treatment for 10 days (days 20–30).

2.2. Photosynthetic and respiration measurements

The photosynthetic capacity of dark adapted (20 min) coral fragments (4 genotypes from each experimental tank) was measured at the end of the study using an imaging pulse amplitude modulated fluorometer (I-PAM, Walz GmbH, Effeltrich, Germany). Rapid light curves (RLC's) were generated by illuminating corals with 0–700 μmol photons $m^{-2} s^{-1}$ (PAR) in 13 steps of 20 s. The maximum photosynthetic efficiency (F_v/F_m) was used in conjunction with non-photochemical quenching values (NPQ) and relative electron transport rate (ETR) to

infer the sensitivity of photosystem II (*PSII*) to changing PAR.

Incubation chambers (300 mL volume) were used to measure rates of dark respiration (Rd) followed by rates of net photosynthesis (Pn) of fragments from each treatment at the end of the study (4 genotypes from each replicate tanks). Coral fragments were immersed in water from the corresponding experimental aquariums with a water change after every measurement. Chambers were made from UV-transparent material with a surrounding flow through water jacket maintained at experimental temperature using a water bath and placed onto a magnetic stirrer to provide mixing. Measurements for both light and dark incubations were conducted for an hour each. During light incubations (net photosynthesis), light was provided by fluorescent lights suspended over the chambers and LED backlights. Light levels at the chamber surface measured ca. 100 μmol photons $m^{-2} s^{1}$. Lights were switched off and chambers were covered by black cloth in order to measure dark respiration. During both net photosynthesis and dark respiration, the concentration of dissolved oxygen (O_2, μmol L^{-1}) was continuously measured inside the chambers using optical probes (Oxy-4, PreSens, Germany) to determine Rd. (in complete darkness) and Pn (in 100 μmol photons $m^{-2} s^{-1}$ light) as μmol O_2 $cm^{-2} h^{-1}$. Rd. and Pn were calculated as: Pn (or Rd) $O_2 = (sO_2 \times V) / S$ where sO_2 is the slope of the linear regression line for change in O_2 over time (μmol $L^{-1} h^{-1}$), V is the volume of the chamber (L) and S is the surface area of the fragment (cm^2).

2.3. Calcification

Total alkalinity (TA) values were measured using an automatic potentiometric titration (Metrohm 862 compact titrosampler) to the second end point (Almgren et al., 1983) of a 32.0-g accurately weighed seawater sample. Water samples were collected after an hour light incubation, filtered (0.2 μm), and were immediately analyzed or stored in darkness at 4 °C. The pH electrodes (Metrohm 862 compact titrosampler) were calibrated daily. The acid titrant concentration was 0.049 N HCl (JT Baker, Phillipsburg, NJ). Total alkalinity values were then computed using the Gran equation (DOE, 1994) with pH values lower than 3.9 for creating the Gran plot. The differences between duplicate samples were < 6 μEq kg^{-1} (for calibration of the titrator, differences were measured among triplicate samples). Calcification values were calculated using the alkalinity anomaly method (Riebesell et al., 2010) and calcification rates were normalized to coral fragment surface area.

2.4. Tissue removal, surface area, protein, and chlorophyll

Subsequent to calcification and oxygen evolution measurements, coral tissue was completely removed using an air brush and filtered seawater (0.2 μm FSW). The skeleton was retained for surface area determination using the aluminum foil technique (Marsh, 1970). The tissue slurry was homogenized for 30 s (DIAX 100 homogenizer Heidolph Instruments GmbH & Co., KG, Schwabach, Germany) and a subsample (100 μL) was taken for total protein analysis using the Bradford protocol (Bradford, 1976). Duplicate samples (4 μL) were mixed with dye reagent (200 μL, Coomassie Brilliant Blue G-250) in a 96-well plate and incubated for 30 min in the dark at room temperature before the optical density was read on a multiscan spectrum spectrophotometer (595 nm, Biotek HT Synergy plate reader). Samples were run against a standard calibration curve of Bovine Serum Albumin and were normalized to surface area. The remaining slurry was repeatedly centrifuged, discarding the supernatant and re-suspending the pellet in 40 mL 0.2 μm FSW between each centrifugation (5 min, 3000 rpm × 2 and 5 min, 5000 rpm × 1). The final pellet was re-suspended in 2 mL 0.2 μm FSW and homogenized before subsamples (1 mL) were taken for chlorophyll *a* and *Symbiodinium* (100 μL) haemocytometer counts (Improved Neubauer). Dividing zooxanthellae cells which had not separated from the parent cell were counted as one. Samples for chlorophyll were centrifuged (10,500 rpm, 30 s) and re-suspended in 1 mL 90% (v:v) acetone. Chlorophyll *a* was extracted for 24 h at 4 °C before spectrophotometric determination (Ultrospec 2100 pro) according to Jeffrey and Humphrey (1975). These data were later normalized to total protein, surface area of each fragment, or zooxanthellae cells.

2.5. Symbiodinium genetic identity

Symbiodinium DNA was extracted at the end of the study using the Wizard® genomic DNA purification kit (Promega, USA). The internal transcribed spacer 2 (ITS2) region of ribosomal DNA was amplified following the protocol of LaJeunesse (2002) with the primers ITSintfor2, ITS-Reverse, and electrophoresis (DGGE) with a 45–80% denaturing gradient and run at 120 V for 13 h. In the resulting unique banding profiles, diagnostic bands were excised, the DNA was re-amplified and then sequenced using an Applied Biosystems 3730XL automated sequencer at the DNA Laboratory at Arizona State University. Sequences were analyzed using Geneious (version 5.5.8) and compared to GenBank submissions.

2.6. Microbial community-level physiological profiling

A random subsample of 4 corals were used to examine microbial shifts from the different treatment scenarios. Each of the four random corals per treatment represented one genotype, and those four genotypes were held constant among treatments. Therefore, a random representative from each of the four genotypes was randomly selected from each of the eight treatments, for a total of 32 corals. A single Biolog EcoPlate (Biolog Inc., Hayward CA) was used for each of 32 corals tested. Biolog EcoPlates consist of a 96-well plate containing 32 different substrates and a tetrazolium dye, resulting in the development of a purple pigment, and subsequent physiological profile, when substrates are metabolized. Coral tissue was removed as described in the coral biology experiment, above. Approximately 25 mL of tissue slurry was normalized to a standard absorbance among all samples using sterile seawater. EcoPlates were inoculated with 150 μL of normalized slurry $well^{-1}$ and incubated at 25°C for 60 h. Plates were read at A_{595} using the microplate reader and Skanit Software 2.4.2 for Multiscan spectrum.

2.7. Statistics

The statistical program R (R Core Team, 2013) was used to examine independent and interaction relationships between treatment variables (pH, temperature, and nutrients). All data were analyzed using randomized block factorial ANOVAs with pH, temperature, and nutrients as independent variables. Genotype was used as the blocking variable. Zooxanthellae cell/host protein (mg), zooxanthellae cell/surface area (cm^2), and calcification were box cox transformed to meet assumptions of parametric tests. After necessary transformations all parametric assumptions were met (tested with Shapiro-Wilk's test for normality and Levene's test for homogeneity of variances). Effect size was calculated as eta^2 using the R package 'sjstats' when significant results were detected (Lüdecke, 2018). A permutational analysis of variance (PERMANOVA) was used to test for differences in carbon source utilization of the bacterial community using the vegan package in R (Oksanen et al., 2013) with pH, temperature, and nutrients as fixed effects and genotype as a blocking variable. Similarity percentages (SIMPER) analysis was used to determine which carbon source contributed to the greatest dissimilarity among treatments when significant differences were detected. Alpha diversity of each sample was quantified using the Shannon-Wiener index and compared using an analysis of variance (ANOVA) after meeting parametric assumptions. Beta diversity among samples within treatments was quantified using the betadisper function in the betapart package in R (Baselga et al., 2013). An ANOVA was used to compare the beta diversity among treatment scenarios and a Tukey

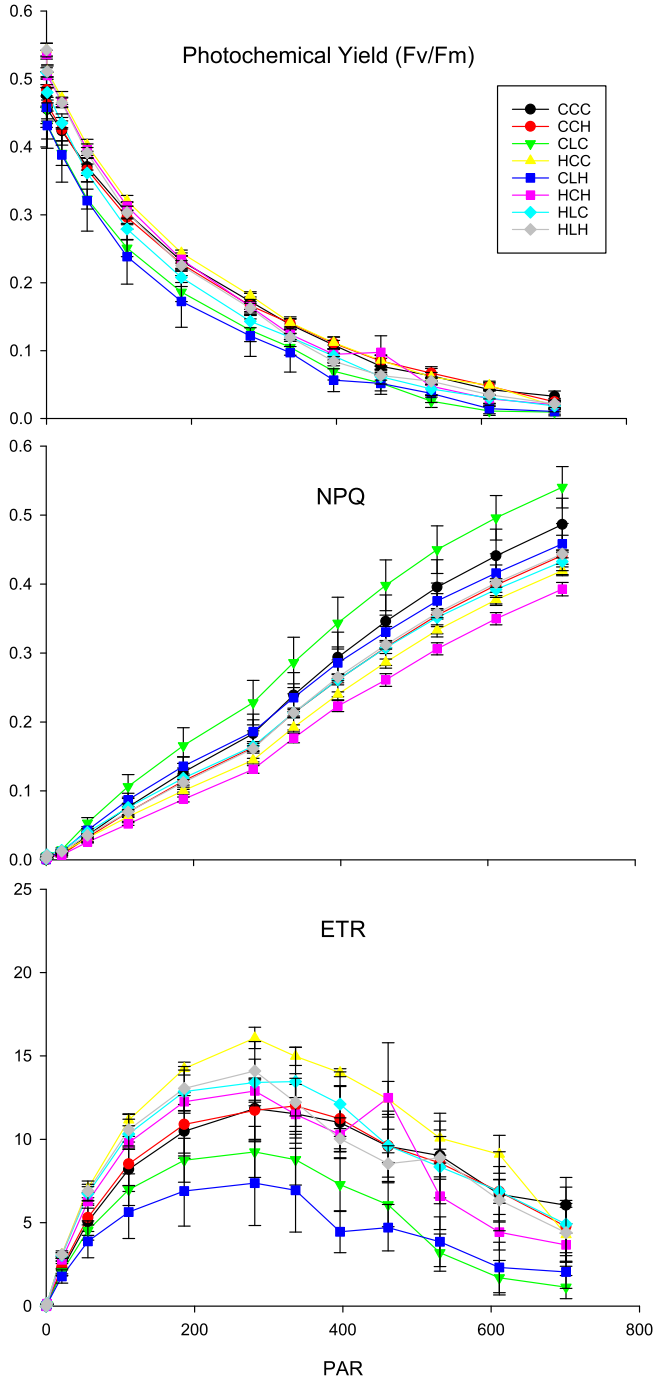

Fig. 1. Chlorophyll fluorescence by PAM for *S. pistillata* including photochemical yield (Fv/Fm), nonphotochemical quenching (NPQ), and relative electron transport rate (rETR). Individual lines are temperature, pH, and nutrient treatments (CCC – control, CCH – high nutrients, CLC – low pH, HCC – high temperature, CLH – low pH + high nutrients, HCH – high temperature + high nutrients, HLC – high temperature + low pH, HLH – high temperature + low pH + high nutrients). Error bars are standard error.

HSD test was used to determine post hoc significant differences among treatments.

3. Results

3.1. Chlorophyll fluorescence, photosynthesis and respiration

Following a 20 min dark acclimation, *S. pistillata* showed

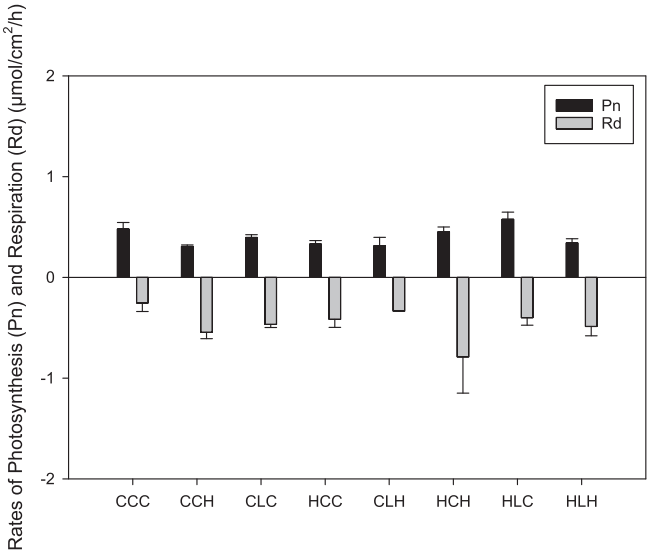

Fig. 2. Photosynthesis (Pn) and respiration (Rd) normalized to surface area (µmol/cm^2/h) in *Stylophora pistillata* after exposure to treatments. CCC – control, CCH – high nutrients, CLC – low pH, HCC – high temperature, CLH – low pH + high nutrients, HCH – high temperature + high nutrients, HLC – high temperature + low pH, HLH – high temperature + low pH + high nutrients. Error bars are standard error.

significantly lower Fv/Fm in the reduced pH ($F_{(1,176)} = 8.20$, $p = 0.005$, eta$^2 = 0.035$) treatment tanks and in the elevated temperature ($F_{(1,\ 176)} = 7.207$, $p = 0.008$, eta$^2 = 0.030$) treatments. The reduced pH treatment also showed increased NPQ relative to control pH ($F_{(1,176)} = 18.768$, $p < 0.001$, eta$^2 = 0.074$). There were no significant differences with ETR (Fig. 1). Nutrients did not significantly affect the photochemical parameters of the tested corals. Additionally, there were no significant interactive effects detected.

S. pistillata showed significantly lower Pn with a combination of elevated temperature, reduced pH, and elevated nutrients ($F_{(1,21)} = 9.305$, $p = 0.006$, eta$^2 = 0.189$) (see Fig. 2). Elevated nutrients also reduced Pn independently ($F_{(1,21)} = 6.325$, $p = 0.003$, eta$^2 = 0.128$). There was a significant interaction between pH and nutrients on Rd. ($F_{(1,21)} = 4.715$, $p = 0.022$, eta$^2 = 0.153$). High nutrients reduced Rd., but only under control pH conditions.

3.2. Calcification

Within the single stressor treatments, decreased pH reduced calcification rates more than increased temperature or increased nutrients alone. However all variables significantly impacted calcification rates (pH: $F_{(1,15)} = 5.13$, $p = 0.036$, eta$^2 = 0.0385$; temperature: $F_{(1,15)} = 15.005$, $p = 0.002$, eta$^2 = 0.109$; nutrients: $F_{(1,15)} = 40.709$, $p < 0.001$, eta$^2 = 0.295$). Additionally, several interactive effects showed significant differences (pH * temperature: $F_{(1,15)} = 16.496$, $p = 0.001$ eta$^2 = 0.119$; temperature * nutrients: $F_{(1,15)} = 10.765$, $p = 0.005$, eta$^2 = 0.078$; pH * temperature * nutrients: $F_{(1,15)} = 27.914$, $p < 0.001$, eta$^2 = 0.202$). Therefore all variables influenced calcification rates, but the nutrients showed the greatest impact. Furthermore, when variables were in combination with nutrients, calcification was much lower (low pH + high nutrients, high temp + high nutrients, high temp + low pH, and high temp + low pH + high nutrients).

3.3. Protein, chlorophyll a, symbiodinium density

There were significantly lower concentrations of protein surface area^{-1} in elevated nutrient treatments than in the ambient nutrient treatments ($F_{(1,21)} = 4.362$, $p = 0.0049$, eta$^2 = 0.121$) (Fig. 3). No

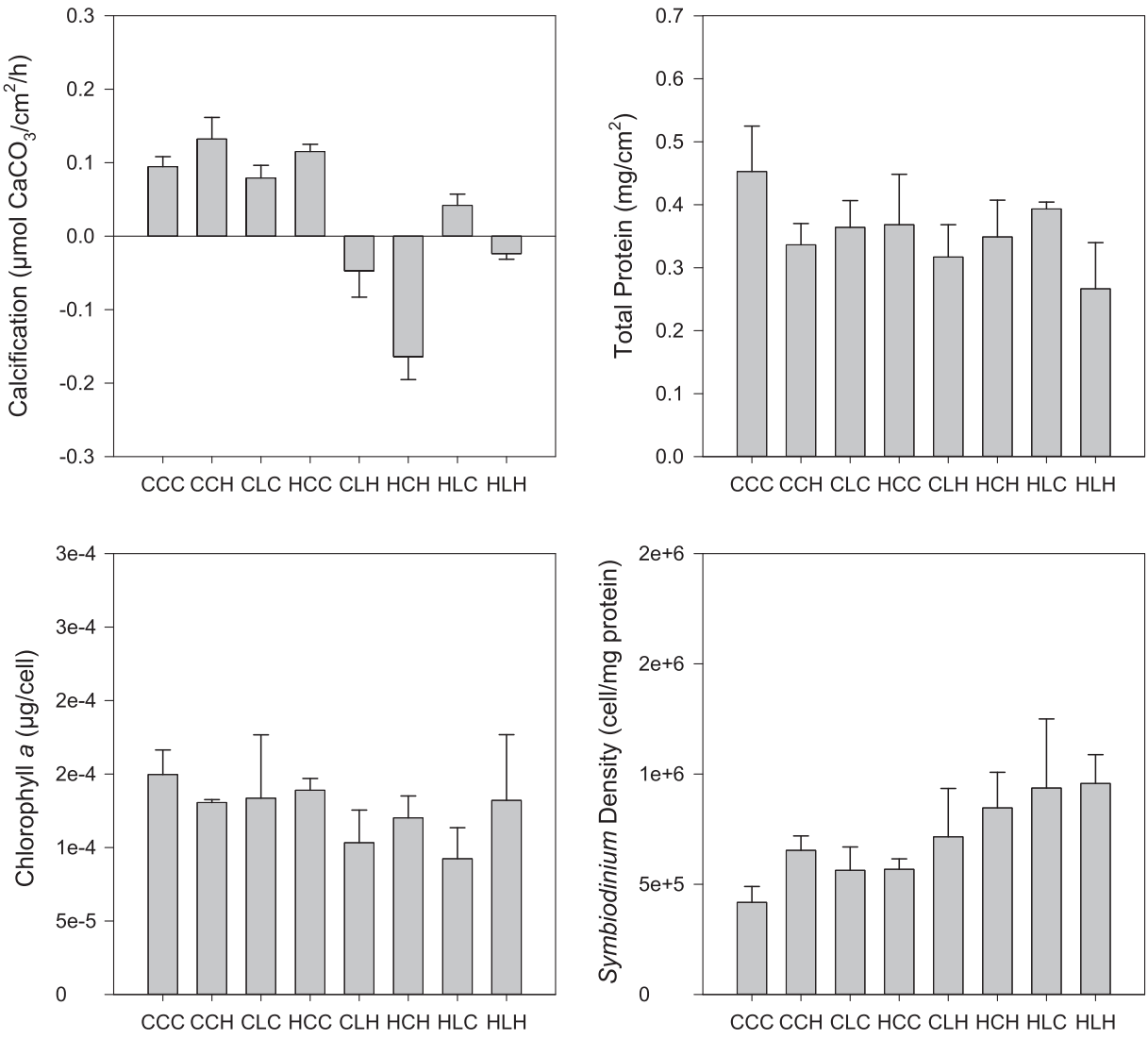

Fig. 3. From left to right, calcification normalized to surface area (µmol $CaCO_3/cm^2/h$), protein concentration (mg/cm^2) normalized to surface area, chlorophyll *a*/zooxanthellae cell, and *Symbiodinium* density (cell/mg protein) in *S. pistillata* after exposure to different treatments. CCC – control, CCH – high nutrients, CLC – low pH, HCC – high temperature, CLH – low pH + high nutrients, HCH – high temperature + high nutrients, HLC – high temperature + low pH, HLH – high temperature + low pH + high nutrients. Error bars are standard error.

other treatment variable showed a significant effect on total protein concentration. There were no significant effects on chlorophyll *a* concentrations (chlorophyll *a*/cell) with any treatment (Fig. 3). *Symbiodinium* density (cells/mg protein) was significantly higher with elevated temperature ($F_{(1,21)} = 6.758$, $p = 0.017$, $eta^2 = 0.163$) and with elevated nutrient ($F_{(1,21)} = 5.001$, $p = 0.036$, $eta^2 = 0.120$) than in any of the controls (Fig. 3). No other interactive or independent treatment variables had a significant effect on *Symbiodinium* density.

3.4. *Symbiodinium* genotypes

The four *S. pistillata* colonies sampled predominantly hosted either clade A *Symbiodinium*, with ITS2 sequences that were identical to the previously described type A1 (accession number AF333505, LaJeunesse, 2001), clade C *Symbiodinium* whose ITS2 sequence was identical to the previously characterized C72 (accession number AY765407, LaJeunesse, 2005) or a mixture of A1 and C72 (Table 1). In addition, one of the fragments of colony 3 hosted a *Symbiodinium* type that is an A1 variant (accession number, EU446105, referred to as IS-113, Correa and Baker, 2009) while another fragment hosted a C type that was one base pair different from the previously identified C15 (accession number JN558044, Pochon et al., 2012).

Table 1
The *Symbiodinium* type found in the eight fragments generated from each of the sampled *Stylophora pistillata* coral colonies.

Colony	No. of fragments with *Symbiodinium* A1	No. of fragments with *Symbiodinium* C72	No. of fragments with *Symbiodinium* A1& C72 or C15*
1	8	0	0
2	7	0	1
3 4	4	2	1,1*
4 6	3	1	4

When looking at the *Symbiodinium* type according to treatment, a pattern emerged. Of the 16 *S. pistillata* fragments exposed to low pH, and either control or high temperatures and control or high nutrients, the vast majority of the fragments, 14, hosted only clade A symbionts, one hosted a mixture of A1 and C72 and one fragment hosted only *Symbiodinium* type C72 (Fig. 4). In the control pH treatment combinations, 3/4 of the fragments in the control temperature, high nutrient conditions hosted *Symbiodinium* type A1. Conversely, 3/4 of the control

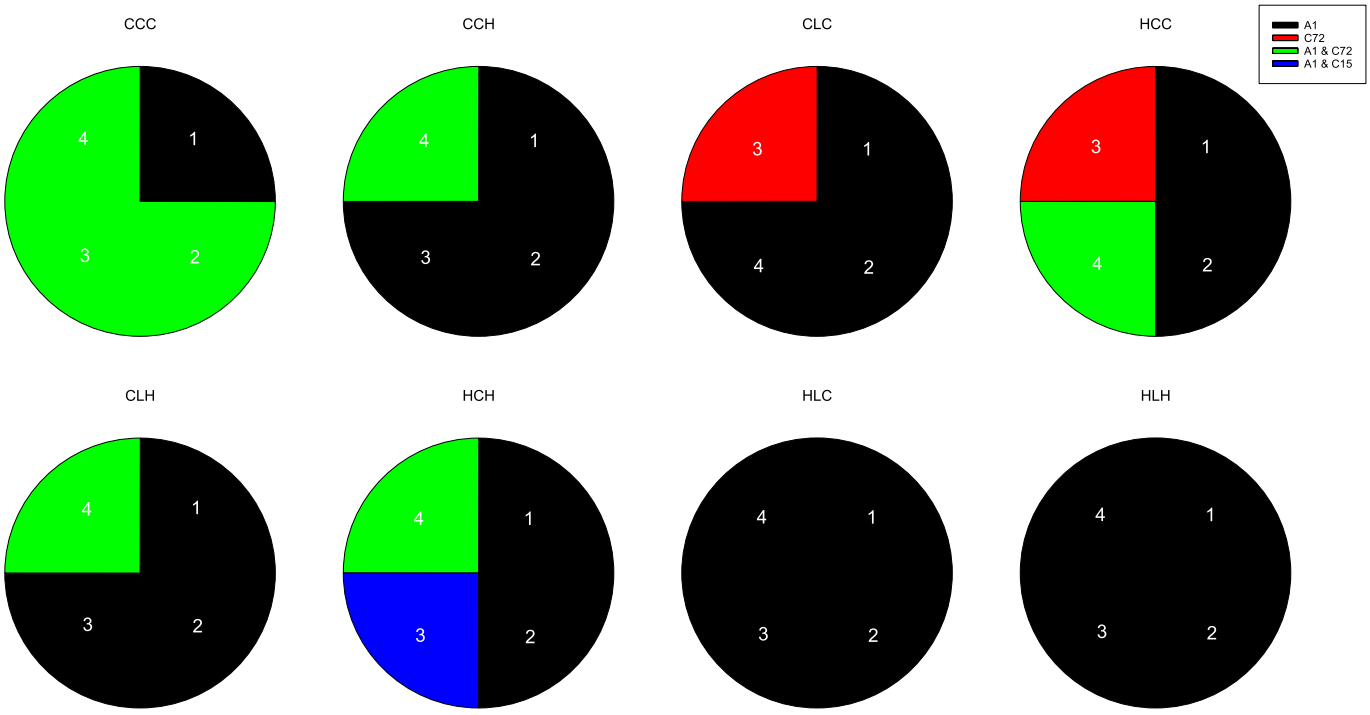

Fig. 4. *Symbiodinium* types found in fragments exposed to combinations of control and reduced levels of pH, and control and high levels of temperature, and nutrients. CCC – control, CCH – high nutrients, CLC – low pH, HCC – high temperature, CLH – low pH + high nutrients, HCH – high temperature + high nutrients, HLC – high temperature + low pH, HLH – high temperature + low pH + high nutrients. Each number refers to colonies 1–4.

temperature, control nutrient fragments hosted a mixture of *Symbiodinium* types A1 and C72. In the control pH and high temperature treatments, half of the fragments hosted A1 *Symbiodinium,* one fragment in each of the nutrient treatments hosted just C72 while the last fragment hosted a mixture of A1 and C72 in the control nutrient conditions and a mixture of A1 and C15 in the high nutrient treatment (Fig. 4).

3.5. Microbial community

Biolog EcoPlate results indicate that there was a significant interaction between temperature and nutrients on microbial communities within the sampled corals ($F_{(1,31)} = 6.117$, $R^2 = 0.155$, $p = 0.03$). The ordination plot shows different distribution patterns especially when comparing the control treatment with corals that experience either high nutrients or high temperature (Fig. 5). Indeed, the single variable conditions (control temperature/high nutrients and high temperature/control nutrients) resulted in less variability among samples (Fig. 5). Additionally, the SIMPER analysis showed the control scenario was the most different from the high temperature and high nutrient scenario (Table 2). In contrast, the single treatment scenarios were the most similar to each other (Table 2). SIMPER analyses indicate that differences observed by temperature and nutrients were primarily driven by utilization of the carbon sources *N*-Acetyl-D-Glucosamine, γ-Hydroxybutyric Acid and D-Cellobiose.

Alpha diversity, measured using the Shannon-Wiener index, did not show significant differences among treatments. However, beta diversity, which measured the diversity of samples within treatments, did significantly differ among treatment scenarios ($F_{(7,24)} = 3.396$, $p = 0.012$). Post hoc Tukey test showed that the beta diversity within control treatment corals significantly differed from those within high temperature, control pH, and control nutrient conditions and from the high temperature, low pH and control nutrient conditions (Fig. 6). This again emphasizes the lack of pH effects, and the low variability within the high temperature and control nutrient treatments within Fig. 5.

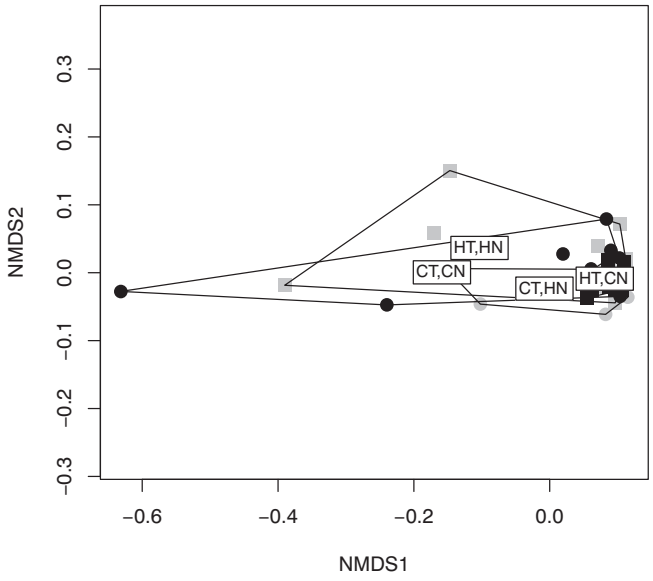

Fig. 5. Ordination plot of the bacterial community within *Stylophora pistillata* exposed to four different temperature (T) and nutrient (N) treatments (C = control, H = high).

4. Discussion and conclusions

Scleractinian corals' fitness is negatively affected by ocean acidification, warming temperatures, and increased nutrients (Anthony et al., 2008; Edmunds et al., 2012; Castillo et al., 2014). Future climate scenarios show that these global and local stressors are likely to occur in combination (IPCC, 2014) which could lead to combined stress on coral fitness. The present study examined the effects of selected forecasted environmental changes both individually and in combination. It is this intricate level of understanding that can most effectively inform local

Table 2
Results of the SIMPER analysis showing the relative dissimilarity between treatment pairs of different temperature and nutrient conditions.

Treatment 1		Treatment 2		
Temperature	Nutrients	Temperature	Nutrients	Relative difference
Control	Control	High	High	0.1846
Control	Control	Control	High	0.1614
Control	Control	High	Control	0.1505
High	High	Control	High	0.1630
High	High	High	Control	0.1607
Control	High	High	Control	0.1025

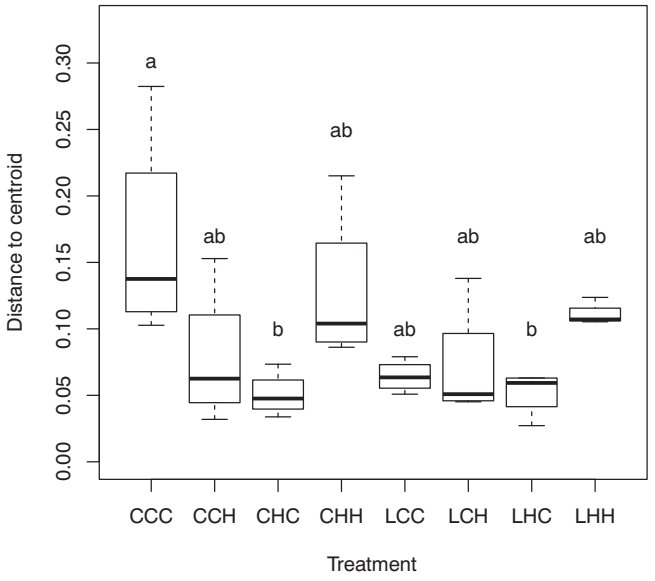

Fig. 6. Box plot of beta diversity for each treatment scenario including pH, temperature, and nutrients exposed to *Styllophora pistillata*. C = control, L = low, H = high.

and regional scale environmental policy makers. The results indicate that in isolation, each of these factors can significantly alter multiple aspects of physiological functioning, and that the effects are often additive when corals are exposed to combination treatments. Specifically, elevated nutrient concentrations had the broadest individual and combination impacts upon the physiology of *S. pistillata* from the Gulf of Aqaba.

The experimental conditions had multiple effects upon the holobiont's energy acquisition. With the rest of the parameters at ambient levels, either decreased pH or high temperature led to significantly reduced photosynthetic efficiency, although they displayed a small effect size (i.e., approximately 3% of the variance of the dependent variable was explained by these factors individually). Increased NPQ was only recorded in reduced pH treatments, with 7% of the dependent variable being explained by pH treatment, considered a medium effect size (Cohen, 1988). Increased NPQ is more typically associated with increased temperature (e.g. Hill et al., 2005; Rodolfo-Metalpa et al., 2006) and the report of increased NPQ under OA conditions is novel. Unfortunately due to the method used, it is not possible to distinguish whether NPQ was the result of photoprotective or inhibitory processes (Maxwell and Johnson, 2000); deciphering the source of significantly increased NPQ under low pH is worthy of further investigation.

Low pH resulted in no other observed significant physiological effects in this study, and when this treatment did cause significant changes there was a small to medium effect size (Cohen, 1988). Even so, decreased photosynthetic efficiency under low pH, combined with the observed increased respiration rates with increased temperature suggests that, in the long term, individual corals' "scope for growth" (Widdows and Johnson, 1988) will likely decrease in future oceans with cascading impacts upon reef growth rate and reproductive output. A comprehensive review of the extant literature indicates that the effect of combined OA conditions with either temperature or nutrients upon zooxanthallae density, oxygen production, and photosynthetic efficiency is often complex or dependent upon additional factors (Ban et al., 2014). For example, despite the common paradigm that OA decreases calcification rate, when OA is experienced in combination with elevated nutrients or temperature, the results from factorial experimental designs are mixed. OA in combination with nutrients can be antagonistic (Holcomb et al., 2010) or additive (Renegar and Riegl, 2005). Likewise, reduced pH in combination with elevated temperature also results in synergistic (Reynaud et al., 2003; Martin and Gattuso, 2009) or additive interactions (Edmunds, 2011; Edmunds et al., 2012). Studies which quantitatively measure calcification most often report no definitive linear trend and/or an insignificant interaction (Ban et al., 2014) as was found in the present study.

A considerable number of studies have assessed the direct effects of elevated dissolved nutrients on coral calcification, tissue growth, and symbionts, and show functionally negative physiological responses (e.g. Hoegh-Guldberg, 1994; Marubini and Davies, 1996; Marubini and Atkinson, 1999; Ferrier-Pages et al., 2000; Fabricius, 2005; Langdon and Atkinson, 2005). However, most experiments were conducted above environmentally realistic levels (Szmant, 2002; Fabricius, 2011). Relevant to this study, the Gulf of Aqaba typically has relatively low concentrations of dissolved inorganic nutrients (Klinker et al., 1978; Korpal, 1991; Al-Qutob et al., 2002; Manasrah et al., 2006; NMP, 2016). However, deep vertical mixing (> 400 m) in the winter causes the local surface nitrate to increase markedly (up to 1.8 μM) (Silverman et al., 2007). Deep vertical mixing introduces nutrient rich water to the otherwise oligiotrophic surface waters. Anomalously high surface nitrate concentrations (NO_3 = 1.2 μM), comparable to those used in the present study, have previously resulted in thick, filamentous, benthic algal blooms that have caused coral mortality (Genin et al., 1995). In addition, this region is occasionally subject to pulses of elevated nutrient loads from anthropogenic land use modifications that can result in flash floods (Kahana et al., 2002). Localized poor water quality has been linked to uncharacteristic algal blooms in the Gulf of Aqaba (Labiosa et al., 2003) and there are reports from both the Gulf of Aqaba (Loya, 2004) and worldwide (Duprey et al., 2016) linking eutrophication to a long-term decline in coral cover and diversity. Elevated nutrients were a driving force for many of the significant physiological responses in this study (reduced protein concentrations, increased *Symbiodinium* density, reduced Pn, and increased rates of respiration when paired with increased temperature). Krueger et al. (2017) also found that *S. pistillata* may be resilient to reduced pH and elevated temperature in the Gulf of Aqaba, yet this study shows that with the addition of nutrients on top of these elevated global stressors, sensitivity is higher, and changes in the symbionts, including bacteria, may occur, leading to a possible shift in the overall holobiont health. These results provide empirical evidence of the deleterious effect of nutrients (N and P) on one of the most abundant Gulf of Aqaba coral species.

Although we observed an increase in *Symbiodinium* density per coral biomass under elevated nutrient concentrations, nutrient enrichment on a regional scale is often associated with increased bleaching events (Fabricius, 2005) and a lowering of coral bleaching thresholds (Wooldridge, 2009). Within the present study we did not observe obvious signs of bleaching, which was also evident by the retention of *Symbiodinium* within the coral tissue and the maintenance of relatively robust maximum photochemical yields under all treatment scenarios. The hypothesized popular mechanism of nutrient induced bleaching cites an unbalanced nitrate: phosphate load (Wiedenmann et al., 2012) which was not the case in our study, as nutrients were added at Redfield ratio. Increased nutrients provide more fertilizer for *Symbiodinium*, so as long as nutrients are balanced, *Symbiodinium* abundance will increase

(Wiedenmann et al., 2012; D'Angelo and Widenmann, 2014). The impact of localized unbalanced nutrient stress in combination with global climate stressors within the Gulf of Aqaba warrants further investigation to assess whether the aforementioned phosphate starvation hypothesis (Wiedenmann et al., 2013) also applies in this region. Our results showed significantly higher respiratory demands under combined low pH and nutrient loading, which may be the cause of decreased protein concentration under high nutrient conditions. As coral colonies deplete their carbohydrate and lipid pools, protein may become the primary respiratory fuel (Lesser, 2013). Using protein concentration as an indicator of energetic reserves leads to the conclusion that corals in elevated nutrients, particularly under low pH conditions, will have a decreased energetic budget and, correspondingly, less plasticity for growth and reproduction.

Overall, in agreement with Nordemar et al. (2003), the results presented here suggest that corals on reefs with nutrient stress will be more vulnerable to global climate change. When nutrient loading was integrated within a treatment the majority showed a large effect size on the dependent variable ($eta^2 = 0.14$ or larger, Cohen (1988)). On top of the physiological traits measured here, nutrient addition has also been linked with increased pathogen growth and virulence (Richardson and Ragoonath, 2008). In addition to direct inputs such as eutrophication and upwelling, nutrient stress is indirectly influenced by many other reef stressors such as flooding, storms, sedimentation, and also pH (Ban et al., 2014). This means that a co-ordinated effort from various management groups will be required in order to limit nutrient stress. The Gulf of Aqaba has been putatively identified as a warming refuge for corals (Fine et al., 2013); although sea temperatures in this region are rising at a similar rate to the global average, a combination of evolutionary history and a variable present day thermal regime, have adapted a range of coral species to be physiologically tolerant of up to 7 °C above mean summer temperature (Fine et al., 2013). Other thermal refugia may exist in cool upwelling zones (Riegl and Piller, 2003), high turbidity reefs (Cacciapaglia and van Woesik, 2016), mesophotic reefs (Riegl and Piller, 2003; Lesser et al., 2009), and reefs with naturally wide thermal ranges (Oliver and Palumbi, 2009). However, the prevalence of such reefs is sparse, making them an even greater conservation priority. A recent assessment concludes that local and regional scale management exerts a significant influence upon the living biomass at reef sites (Kennedy et al., 2013; Cinner et al., 2016). The understanding that corals within the Gulf of Aqaba, while being a rare refuge to the spiraling consequences of global climate change, may in fact be vulnerable to direct and indirect nutrient stress should act as a warning to prevent the addition of local stressors in this region. Borell et al. (2013) demonstrated that macro algae from the GoA which were incubated in higher pCO_2 had lower palatability to the natural grazing fish community in the Eilat reef (Borell et al., 2013). Reduced grazing may lead to macro algae dominance in an increased nutrient and pCO_2 environment regardless of the coral performance.

Coral cores from *Orbicella faveolata* provide evidence of significantly more historical bleaching events at Mesoamerican reef sites with greater chronic human disturbance levels (sedimentation, nutrient input, population size, and fishing pressure), together with lowered bleaching resistance and longer recovery times (Carilli et al., 2009.). In addition, although water temperatures were lower in this region than other years over the past century, modern pressures resulting from an expanded human population resulted in unprecedented coral bleaching in 1998 (Carilli et al., 2010). This implies that reefs formally resistant to high temperature stress may not withstand warming and/or acidification when combined with synergistic, local scale pressures such as eutrophication and sedimentation. Correspondingly, modeling future warming and terrestrially-sourced dissolved organic nitrogen loading scenarios onto the near-shore Great Barrier Reef, indicates that reducing ambient end of river dissolved inorganic nitrogen (DIN) by > 50% would equate to a 2.25–2.50 °C increase in bleaching threshold (Wooldridge, 2009). *S. pistillata* may acclimatize to prolonged exposure (9 weeks) to high nutrients (ammonium and phosphate) as indicated by increased growth rates and production to respiration ratio with time, however, these adjusted rates likely remain lower than corals low nutrient environs (Ferrier-Pagès et al., 2000).

Scleractinian corals can host either a single *Symbiodinium* or multiple *Symbiodinium* types (Goulet, 2006, 2007). *S. pistillata* is one of the coral species capable of hosting two *Symbiodinium*. For example, on the Great Barrier Reef, it hosts *Symbiodinium* belonging to clades C and D (Sampayo et al., 2008), while in the Gulf of Aqaba, it hosts *Symbiodinium* belonging to clades A and C (Winters et al., 2009; Byler et al., 2013). In the Winters et al. (2009), the *S. pistillata* colonies sampled had either *Symbiodinium* A or C and the *Symbiodinium* present correlated with depth, with A in shallow and C in deep. Byler et al. (2013) found that in their sampled colonies, *S. pistillata* from > 20 m depth hosted not only *Symbiodinium* type C72 but some also had both type A1 and C72 simultaneously. Of the 32 coral fragments used in our study (eight fragments from each of four shallow water colonies) 29 fragments indeed had A1 *Symbiodinium*, but in addition, 24% of these fragments also hosted detectable levels of *Symbiodinium* C72, with four out of these seven fragments belonging to the same coral (Table 1). Since the DGGE technique may not pick up *Symbiodinium* if they comprise 5–10% of the *Symbiodinium* population (Thornhill et al., 2006), the C72 *Symbiodinium* detected in the *S. pistillata* fragments constituted > 10% of the *Symbiodinium* population. The remaining three fragments, two from the same colony, hosted only C72 at detectable levels. Therefore, our study adds on to Byler et al.'s (2013) study, further reiterating that *S. pistillata* can host two *Symbiodinium* types simultaneously.

Similar to other coral species that can host two *Symbiodinium* types within the same coral colony simultaneously (Berkelmans and van Oppen, 2006), it is possible that in *S. pistillata* a shuffling of the *Symbiodinium* types occurs in response to environmental conditions. In the control pH conditions 50% of the fragments hosted *Symbiodinium* C. On the other hand, of the 16 coral fragments in aquaria with low pH conditions, all but one displayed A1 *Symbiodinium* at detectable levels, with only two fragments hosting C72 either singly or with A1. Within *S. pistillata*, *Symbiodinium* A withstands harsher environmental conditions compared to C (Winters et al., 2009). Potentially, the lower pH conditions are more conducive for clade A *Symbiodinium*. Likewise, under ambient pH conditions and temperature, but comparing ambient versus high nutrient conditions, only one fragment in the high nutrient conditions hosted C *Symbiodinium* in addition to A while in the ambient nutrient conditions three of the four fragments contained both A and C. On the other hand, at ambient pH but elevated temperature, nutrient levels did not have a dramatic effect on the *Symbiodinium* in the fragments, attesting to the complex outcome of multiple stressors.

Bacterial communities within corals are known to shift under high temperature conditions (Ritchie, 2006). High temperature conditions can also change bacterial behavior, perhaps leading to higher probability of disease occurrence (Garren et al., 2016). Nutrients can also change bacterial community structure, and high levels of nutrients may cause increased mortality rates (Zaneveld et al., 2016). Bacterial communities may be altered by pH as well, although some studies show tolerance to changes in pH (Muller et al., 2016) or indicate mixed results (Webster et al., 2013; Webster et al., 2016). Microbes are ubiquitous and are often the first organisms to respond to subtle chemical and physical environmental changes. Microbial community-level profiling using Biolog Ecoplates is a rapid, cost effective tool used to provide information regarding chemical and physical changes (Radi et al., 2005). Most microbes are unable to be cultured away from their preferred niche, including associated hosts, habitats and microbial partners. An additional advantage of the Biolog method is that microbial samples can be inoculated directly into plates without an isolation stage and without altering any component of the microbial community of the coral. Biolog Ecoplates also provide an effective tool for comparing functional diversity of whole microbial communities. Microbial profiling using this method showed a significant interactive effect of

temperature and nutrients on microbial communities based on metabolic usage shifts, with no observable effects related to pH (Fig. 5). The SIMPER analyses also showed that the control treatment and the high temperature and high nutrient treatment displayed the most dissimilar bacterial community, a result of significant shifts in community structure. Furthermore, the two treatments that were most similar with regards to microbial metabolic profiles were the control temperature/high nutrients and the high temperature/control nutrient treatment. These results suggest single treatments effects may cause less of a shift in microbial communities and multiple stressors could have an additive effect when structuring the bacterial community. The greater dissimilarity between controls and the control temperature/high nutrient treatment, compared with the high temperature/control nutrient treatment suggests nutrients may have a stronger immediate impact on the microbial community composition than temperature.

The primary carbon sources, that contributed the most to dissimilarity among treatments was *N*-Acetyl-D-Glucosamine (NAG), γ-Hydroxybutyric Acid and D-Cellobiose. *N*-Acetyl-D-Glucosamine is a monosaccharide, a derivative of glucose, and a critical component of bacterial cell walls, and a provider of both carbon and nitrogen as nutrients (Sala et al., 2006). Previous studies suggest that much of the bacteria that utilize NAG are facultative anaerobes, which can use fermentation as a form of metabolism (Riemann and Azam, 2002). γ-Hydroxybutyric Acid is a carboxylic acid and D-Cellobiose is a disaccharide that can be hydrolyzed to glucose enzymatically or with acid. Both contribute carbon solely as nutrients. Although it is difficult to determine the overall bacterial community structure from the differential utilization of these particular nutrient sources, differences detected from the Biolog Ecoplates indicate, not only that the bacterial community indeed changes, but that the functional diversity of the community is altered under different environmental scenarios.

The interactive effects of stressors are spatially variable and therefore reefs should be managed on a local scale in accordance with locally obtained scientific data. While Hughes et al. (2017) argue that overfishing and water quality is not a factor affecting the resilience of reefs on the Great Barrier Reef; this is not likely the case in the Gulf of Aqaba. The Great Barrier Reef is a larger reef area so there is greater dispersal and likely less anthropogenic impact from nutrient run-off. The Gulf of Aqaba reef is fringing along a highly populated coast where flash floods bring in heavy use of fertilizers and pollutants such as oil into a very concentrated enclosed space. The present study is among few studies that report on a factorial combination of three contemporarily relevant stressors. In addition, these data provide a critical and specific knowledge advancement to aid the conservative management of a locally dominant reef building species in the Gulf of Aqaba. In conclusion, while Gulf of Aqaba corals may be resilient to OA and warming (Fine et al., 2013; Osman et al., 2017; Roik et al., 2017), a rise in nutrient concentrations would severely impede the reef's capacity for growth and significantly alter the bacterial community structure and functionality within the coral holobiont. Therefore management strategies that reduce local stressors will be required to facilitate the Gulf of Ababa's corals' potential to survive future climate change.

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